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EFFECT OF NONADECAFLUORODECANOIC ACID ON MICROSOMAL STEAROYL-CoA DESATURASE AND ELECTRON TRANSPORT ACTIVITIES IN RAT LIVER

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

ROGER C. INMAN, Colonel, USAF
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The biochemical basis of the hepatotoxicity of NDFDA (nonadecafluorodecanoic acid) has been investigated from the point of view of its effects on stearoyl-CoA desaturase and associated electron transport functions in microsomes. Changes in these parameters would be consistent with earlier observations on altered liver fatty acid content in NDFDA-treated rats. In these preliminary studies, hepatic stearoyl-CoA desaturase activity dropped in both NDFDA-treated rats and in pair-fed controls, approaching zero within 6-8 days. This decrease was attributed mainly to decreased food intake and did not appear to be

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directly related to NDFDA hepatotoxicity. However, NDFDA induced several
biochemical changes not present in the pair-fed controls. These included (1)
decreased inducibility of stearoyl-CoA desaturase by force feeding an amino
acid/sucrose mixture, (2) a marked decrease in the rate of microsomal electron
transport from NADH through cytochrome b_5 to the terminal oxidases (including
the desaturase) and molecular oxygen, and (3) an increase in the concentration
of cytochrome P-450, an important component of the microsomal drug metaboliz-
ing system. NDFDA caused a small decrease in microsomal cytochrome b_5. Serum
glucose levels were normal in NDFDA-treated rats, suggesting that insulin
levels were also normal. The effects of NDFDA on the liver appear to reflect
direct or indirect modulation of gene expression and/or specific changes in
membrane systems rather than non-specific cellular damage.

PREFACE

This report describes one of a series of related on-going studies on the toxicity of nonadecafluorodecanoic acid (NDFDA) in the Biochemical Toxicology Branch, Toxic Hazards Division, Air Force Aerospace Medical Research Laboratory. The thrust of this particular study was investigation of microsomal enzyme activities in NDFDA-treated rats. Investigations of the hepatic metabolism of xenobiotic chemicals or of the effects of these chemicals on hepatic enzymes are supported by Task 630202, "Toxicokinetics and Pharmacodynamics of Air Force Chemicals;" Work Unit 63020215, "Physiological-Toxicokinetic Modeling of Inhalation Exposure." The work described in this report was carried out during the period from 1 June to 31 July 1982. T. E. Webb was a Fellow in the Summer Faculty Research Program conducted by the Southeastern Center for Electrical Engineering Education (SCEEE) under Air Force Contract No. F49620-82-C0035.

Nonadecafluorodecanoic acid (NDFDA), a straight chain perfluorinated 10 carbon acid ($CF_3(CF_2)_8CO_2H$), is similar in structure to compounds used as film forming foam fire extinguishants. These polyfluorinated chemicals are also used to impart oil and water resistance to porous materials. NDFDA is extremely toxic and may serve as a model compound for evaluating the health hazards associated with other perfluorinated compounds used by the Air Force, other Armed Forces, and the general public. To evaluate the health hazards of NDFDA, it is necessary to elucidate the mechanism of its toxicity.

In previous studies at AFAMRL (Andersen et al., 1981a.b.; Bacon et al., 1981; Olson, 1982) NDFDA dosages in the region of the LD_{50} (i.e., 50 mg/kg body weight) were found to cause anorexia and rapid weight loss in rats. Pathological changes were noted mainly in the liver, but also in the bone marrow, thymus, stomach, and testes. Changes also occurred in the membranes of red blood cells from NDFDA-treated rats[1] and also in the concentration of rat liver fatty acids (Olson, 1982) which serve as precursors for membrane lipids and phospholipids. In particular, the elevation of oleic acid and diminution of stearic acid in the livers of NDFDA-treated rats focused attention on stearoyl-CoA desaturase, the enzyme involved in the desaturation reaction which converts stearic to oleic acid.

The main objectives of this study were to determine the effects of NDFDA on hepatic stearoyl-CoA desaturase activity, on associated microsomal electron transport components of this desaturase system (including cytochrome b_5 and cytochrome b_5 reductase), and on the alternative microsomal terminal oxidase, cytochrome P-450. The conversion of stearic to oleic acid by stearoyl-CoA desaturase which requires NADH and molecular oxygen occurs in liver microsomes according to the following scheme:

$$NADH \longrightarrow NADH\ cyt\ b_5\ reductase \longrightarrow cyt\ b_5 \searrow$$

$$stearate\ desaturase\quad +\quad O_2$$

$$stearoyl\text{-}CoA \longrightarrow oleate$$
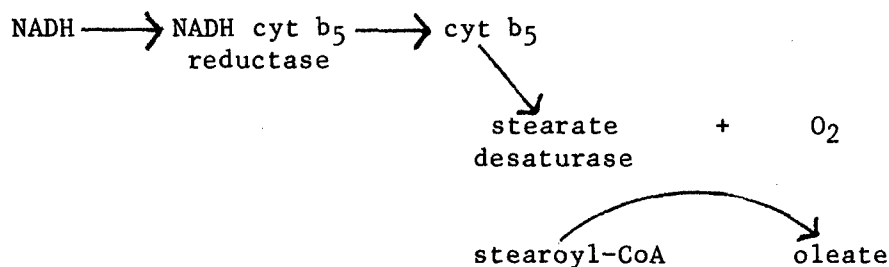
In the absence of stearoyl-CoA, electrons from NADH may be transferred via cytochrome b_5 to cytochrome P-450, an alternative terminal oxidase involved in drug metabolism and which, in the absence of substrate, undergoes rapid autooxidation (Ivanetich et al., 1980; 1981).

Because stearoyl-CoA desaturase is sensitive to nutritional status and insulin levels in animals, appropriate controls were included in this study. These are so-called pair-fed controls in which control rats are fed only as much food as treated rats have consumed. Pair feeding is done by

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[1] Andersen, M. E. and George, M. E., 1982, unpublished observations

measuring food intake in treated rats for 24 hr and then giving that amount of food to control rats for the next 24 hr period. In addition, we also tested for the inducibility of stearoyl-CoA desaturase in vivo. In a normal rat the desaturase activity can be increased 2 to 3 fold by a regimen in which the rat is first fasted, then force-fed a synthetic diet, and the desaturase activity determined 15 hr after force feeding. The capacity for induction is a measure of the integrity of regulatory control mechanisms in the liver.

MATERIALS AND METHODS

Materials and Animals

Nonadecafluorodecanoic acid (Lot #0104BE; 96-100% pure) was purchased from Aldrich Chemical Company, Milwaukee, Wisconsin. The assay reagents, NADH and stearoyl-CoA, were obtained from the Sigma Chemical Company, St. Louis, Missouri and P-L Biochemials, Milwaukee, Wisconsin, respectively. Male Fischer 344 rats were obtained from Charles River Company. They were fed commercial rat chow and used for experiments when they weighed between 180 and 220 g. Force-feeding by stomach tube was carried out under light halothane anesthesia.

Experimental Protocol

Since NDFDA treated rats have drastically reduced food intake, pair-fed controls were run. In a typical experiment, 8-10 rats were injected intra-peritoneally (ip) with a single dose of 50 mg of NDFDA/kg with propylene glycol:water (1:1; v/v) as vehicle. Livers were harvested from 2 rats at each time interval: 2, 4, 6, and 8 days after NDFDA injection. Liver samples were taken one day later from pair-fed controls injected with the vehicle. In some experiments one NDFDA-treated and one control rat were force-fed by intubation with 5.0 ml of an aqueous solution (pH 7.0) of 20 g % sucrose and 20 g % Bactotryptone (a pancreatic enzyme digest of casein), 15 hours before sacrifice. With selected animals, blood was removed just prior to removal of the liver and serum glucose was determined in the Pathology Branch of the Toxic Hazards Division.

Preparation of Microsomal Fractions

The microsomal fraction was purified from livers as described by Strittmatter et al. (1972). Briefly, the liver was perfused via the portal vein with 0.25 M sucrose prior to surgical removal and 2.5 gm aliquots were minced and homogenized in 22.5 ml of a 0.75 M sucrose buffer (1.0 mM EDTA-10 mM Tris-acetate, pH 8.1) using a glass homogenizer with a rotating teflon pestle. The homogenate was centrifuged for 15 min at 18,000 g in a Sorvall RC2B centrifuge and the post-mitochondrial supernatant was recentrifuged under identical conditions. To isolate the crude microsomal fraction, the recentrifuged post-mitochondrial supernatant was centrifuged at 120,000 g for 30 min in a swinging bucket rotor (SB 283) in an IEC ultracentrifuge. Microsomal pellets were washed by resuspension in 0.5 M NaCl-0.1 M

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Tris-acetate buffer, pH 8.1, then recentrifuged at 120,000 g for 30 min as described above. After a second wash in 0.1 M Tris-acetate, pH 8.1, purified microsomes were suspended in the same buffer to give a protein concentration of approximately 2.0 mg protein/ml. (Concentrations in this range were achieved upon resuspension of microsomes from 1.25 gm of liver in 3.0 ml of 0.1 M Tris buffer.) To assure total resuspension of the microsomal pellet after each ultracentrifugation, the pellet was initially dispersed in buffer by vortexing, then uniformly resuspended by gentle hand homogenization using a small glass homogenizer fitted with a tight teflon pestle.

All of the above procedures were quickly carried out at 2-4°C and the microsomal suspension was stored in ice for no more than 3 hours before use. A light sonication (10-15 sec) of the final microsomal suspension, followed by a 5 min centrifugation at 2000 rpm, also helps reduce interference in the spectrophotometric determinations. Aliquots (0.10 ml) of each microsomal suspension were stored at -20°C for protein analyses with the Folin reagent (Lowry et al., 1951).

Desaturase Assay

The spectrophotometric assay for microsomal stearoyl-CoA desaturase activity, described by Strittmatter et al., (1974), was adapted for measurement by a microprocessor-controlled Gilford Model 2600 spectrophotometer. The assay involves detection of the onset of cytochrome b_5 reoxidation in a microsomal suspension at a wavelength of 424 nm after reduction with 1.0 nmole of NADH in the absence (B) and presence (A) of excess stearoyl-CoA at 30°C (Fig. 1). Cytochrome b_5 is very rapidly reduced by NADH through the mediation of cytochrome b_5 reductase and remains reduced until all of the NADH is oxidized by electron transfer through cytochrome b_5 to oxygen. Part of the autooxidation is accomplished via cytochrome b_5 and the desaturase, but mainly by electron transfer to other terminal oxidases such as cytochrome P-450 (Ivanetich et al., 1981). Once all the NADH is oxidized, cytochrome b_5 becomes reoxidized and the absorbance at 424 nm (A_{424}) decreases. In the presence of stearoyl-CoA, electrons are also transferred from cytochrome b_5 to the desaturase and the oxidation of NADH occurs more rapidly. Assuming that 1.0 mole of NADH is required for each mole of oleyl-CoA formed, the activity in nmol/min/mg protein is (B-A)/(B x mg protein), where B and A are the times to onset of reoxidation of cytochrome b_5 in the absence and presence of stearoyl-CoA, respectively.

Reactions were carried out in a teflon stoppered, 0.4 ml microcuvette, with a 1.0 cm light path. The initial reaction mixture typically consisted of 0.15 ml of microsomal suspension and 0.15 ml of 0.1 M Tris-acetate buffer, pH 8.1. Following temperature equilibration, 1.0 μl of freshly prepared solution containing 1.0 nmoles NADH/μl was added, the contents of the cuvette were rapidly mixed by inversion, and the time scan at 424 nm was initiated. Following complete oxidation of the NADH and reoxidation of cytochrome b_5, 3.0 μl of a solution containing 2.0 nmoles stearoyl-CoA/μl and 1.0 μl of the NADH were added in rapid succession. The contents were once again mixed by inversion and then scanned at 424 nm as above. (All substrates were prepared in 0.1 M Tris-acetate buffer, pH 8.1. The stock stearoyl-CoA solution can be stored frozen.) By extrapolating the maximum

rate of oxidation of cytochrome b_5, the time for reoxidation of NADH is estimated for the dual scans and stearoyl-CoA desaturase activity is calculated as nmoles/min/mg protein, as outlined above.

NOTE: Although halothane, used in these studies as an anesthetic, is known to enhance electron transfer from cytochrome b_5 in vitro (Ivanetich et al., 1980), millimolar concentrations are required as compared to trace amounts accumulating during anesthesia. In addition, most of the halothane in the liver sample will be lost during preparation of the microsomes.

Estimating Cytochromes b_5 and P-450

The nmoles of cytochrome b_5/mg microsomal protein were estimated directly from the decrease in A_{424} on reoxidation of cytochrome b_5 using a millimolar (E_{mM}) extinction coefficient of 100 (Strittmatter et al., 1974). The data were recalculated as % of cytochrome b_5 in liver microsomes from a normal fed rat after correcting all samples to a standard protein concentration of 1.0 mg/ml.

The concentration of microsomal cytochrome P-450 was estimated by the difference spectrum of its carbon monoxide derivative form. Since carbon monoxide binds only the reduced heme protein, cytochrome b_5 is first reduced with sodium dithionite prior to exposure to carbon monoxide. These assays were carried out in two stoppered cuvettes (3-4 ml) with a 1.0 cm light path. Each cuvette contained equal amounts of microsomes (ca 2.0 mg protein/ml) in 0.05 M phosphate-1.0 mM EDTA buffer, pH 7.6, and sodium dithionite was added to both. Carbon monoxide was bubbled through the microsomal suspension in one of the cuvettes for two, 20 sec treatments as specified by Maizel (1971). The Gilford 2600 spectrophotometric system scanned each sample between 400 and 500 nm, then plotted the difference spectrum directly. Using a millimolar extinction coefficient of 91 (E_{mM}), the nmoles of cytochrome P-450/mg protein was calculated from the relation.

$$\text{nmoles cytochrome P-450} = \frac{A_{450-480} \times 1000}{91 \times \text{mg protein}}$$

An indirect (semiquantitative) assay of NADH-cytochrome b_5 reductase was made in the present study. This enzyme is normally present in liver microsomes in excess so that cytochrome b_5 is reduced almost instantaneously upon addition of NADH. Initial portions of time scans (cf. Fig. 1) were checked to see if the reduction phase was prolonged. This would have indicated that the concentration of this enzyme had markedly decreased, i.e., became rate-limiting.

RESULTS AND DISCUSSION

A summary of the changes in stearoyl-CoA desaturase activity in NDFDA-treated animals and their pair-fed controls and the inducibility of
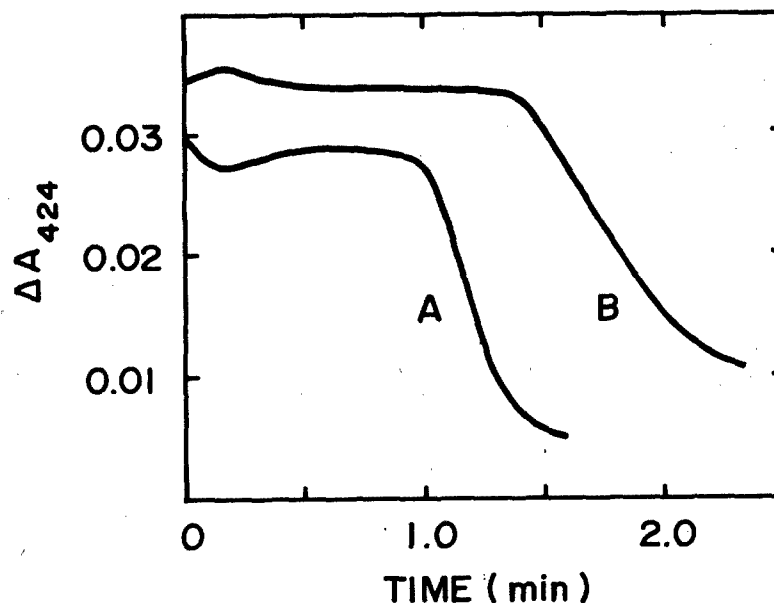
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Figure 1. Change in A_{424} as a function of time during the reoxidation of 1.0 nmole of NADH by a microsomal suspension from a normal fed rat in the absence (B) and presence (A) of excess stearoyl-CoA. In this example A and B were estimated to be 1.00 and 1.425, respectively.

the enzyme by dietary means is shown in Figs. 2a and 2b as a function of time post-treatment. The enzyme decreased dramatically in both groups of animals, the activity approaching zero within 4-5 days. However, most of the loss of activity can be attributed to decreased food intake, which declined in a roughly linear fashion in the NDFDA-treated rats and was essentially zero by 6 days post-treatment.

In contrast, a real difference was observed between the NDFDA-treated rats and their pair-fed controls when enzyme inducibility was assessed. In these experiments the treated or pair-fed rats were force-fed (intubated by stomach tube) with the sucrose:casein hydrolysate mixture 15 hours before sacrifice. In the pair-fed controls the fasting imposed by the pair feeding regimen acted in concert with the 15 hour forced feeding to induce the desaturase activity to levels twice those found in the liver of a normal fed rat. In contrast, only slight induction was observed during the earlier phase of the experiment in the livers of NDFDA-treated rats. Inducibility was absent 8 days after NDFDA treatment.

Since stearoyl-CoA desaturase activity is inducible by insulin and may require insulin for dietary induction (Prasad and Joshi, 1974), the insulin status of these animals was indirectly determined by measuring blood glucose levels in serum samples from selected rats. Glucose concentrations were not significantly higher in NDFDA-treated rats indicating that insulin levels were within the normal range in these animals (Table 1).
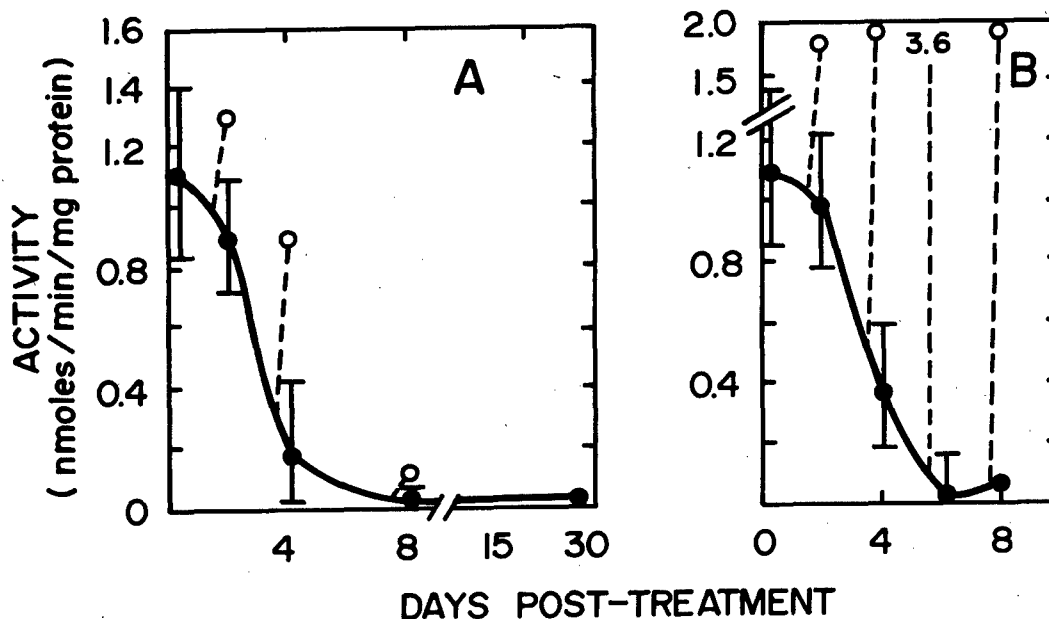
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Figure 2. Time-course changes in the activity of stearoyl-CoA desaturase activity in the livers of (A) rats treated with NDFDA or (B) pair-fed controls as a function of duration of post-treatment (●——●). The 15 hour time-course of induction in response to forced feeding is also shown (o---o). In some instances where there was no stearoyl-CoA desaturase activity in the microsomal preparation, the addition of stearoyl-CoA actually delayed further the oxidation of NADH.

Table 1. Serum Glucose Levels in Normal and NDFDA-Treated Rats

Treatment	Serum Glucose* (mg/dl)
Normal fed rat	151, 137
NDFDA-treated rat 8 days post-treatment	118, 142, 136
NDFDA-treated rat 8 days post-treatment 15 hrs after force-feeding	92, 117, 125

* Individual values are shown.

Pretreatment of rats with NDFDA resulted in a significant (2-3 fold) decrease in the rate of reoxidation of NADH by the microsomal fraction in the standard stearoyl-CoA desaturase assay (Table 2). This delay was observed both in the absence and presence of stearoyl-CoA. However, the rate of oxidation of NADH by microsomes from the pair-fed controls was similar to that of microsomes from normal fed rats. Force-feeding of the

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Table 2. Time for Oxidation of NADH by Microsomes from
NDFDA-Treated, Pair-Fed, and Normal Rats

Treatment	Time for Oxidation of 1 nmole NADH (min/0.3 mg protein)	
	NADH Only	NADH + Stearoyl-CoA
Normal fed rats	1.57 ± 0.40	1.30 ± 0.45
NDFDA-treated rats 6-8 days post-treatment	4.58 ± 0.35	4.29 ± 0.38
NDFDA-treated rats Force fed	4.59 ± 0.33	4.50 ± 0.64
Pair-fed rats	1.31 ± 0.30	1.24 ± 0.11
Pair-fed rats Force fed	1.28 ± 0.45	1.23 ± 0.11

animals did not alter these differences. Since the reduction of cytochrome
b_5 by NADH appeared to be very rapid in all assays (i.e., was not observable
under our conditions of assay), the cytochrome b_5 reductase appears to be
present in excess in both the NDFDA-treated and pair-fed animals. The
modification in the microsomal fraction which results in increased time for
reoxidation must be distal to cytochrome b_5, the oxidation of which is also
unchanged. Since the effect is observed in both the presence and absence of
stearoyl-CoA, it must also be proximal in the pathway to the terminal
oxidases.

Table 3. Effect of NDFDA Treatment on
Liver Microsomal Cytochrome b_5 Content in Rats

Treatment	Cytochrome b_5 Content (% Normal Fed Control)*
NDFDA-treated	
2 days	83.2
4 days	75.0
8 days	72.1
NDFDA-treated/force fed	
2 days	88.0
4 days	82.0
8 days	77.1
Pair-fed controls	
2 days	86.7
4 days	91.5
8 days	107.3
Pair-fed/force fed	
2 days	149.9
4 days	112.5
6 days	90.4

* The microsomes from the normal fed control rat
had 0.974 nmoles of cytochrome b_5 per mg protein.

The liver microsomal cytochrome b_5 content in individual rats is summarized in Table 3 as % of cytochrome b_5 in liver microsomes from a normal fed rat. The concentration was estimated from the decrease in A_{424} incident to the reoxidation of NADH-reduced cytochrome b_5 in the standard desaturase assay (cf. Fig. 1). In general, there was no consistent change in the microsomal cytochrome b_5 content of liver from NDFDA-treated rats. Even the lowest value observed 6 days post-treatment was still 75% of the normal control value. Further, there were no large differences between the treated and treated/force fed groups. The decrease in cytochrome b_5 content was somewhat less in the pair-fed controls and was elevated above the normal control values when these animals were force-fed the sucrose:casein hydrolysate mixture. Changes in cytochrome b_5 cannot account for changes in the rate of either oleic acid production or microsomal electron transport observed in the microsomal preparations from treated rats.

Cytochrome P-450 may act as terminal oxidase for the reoxidation of NADH reduced cytochrome b_5 in the absence of stearoyl-CoA desaturase (Ivanetich et al., 1980, 1981). The concentration of cytochrome P-450 in liver microsomes was estimated by the difference spectrum of its carbon monoxide derivative (Table 4). These limited data suggest that cytochrome

Table 4. Effect of NDFDA Pretreatment on Liver Microsomal
Cytochrome P-450 Content

Treatment	Cytochrome P-450 (% Normal Control)
NDFDA-treated rats	
4 days	178.9
6 days	100.0
Force-fed NDFDA-treated rats	
4 days	212.3
6 days	175.4
Pair-fed controls	
4 days	31.75
6 days	72.98
Pair-fed, force-fed controls	
4 days	49.1
6 days	81.7

P-450 levels in the liver were increased slightly after NDFDA. This increase is even more significant in view of the decreased cytochrome P-450 in the microsomes of the pair-fed controls. In any case, these results rule out the possibility that decreased microsomal transport in NDFDA-treated rats is due to a decrease in this particular terminal oxidase.

CONCLUSIONS AND RECOMMENDATIONS

The spectrophotometric method employed in this study to assay microsomal stearoyl-CoA desaturase depends upon the integrated, sequential functioning of several enzymes and electron acceptor proteins which are membrane components. The results of the present study indicate that both the overall desaturase activity and the rate of microsomal electron transport are reduced in the NDFDA-treated rats. Due to the complexity of this enzyme system, it would be desirable to obtain a more direct estimate of the rate of conversion of stearic acid to oleic acid in vivo. This could be done in an experiment in which labeled stearic acid is injected via the portal vein. After several short time intervals, the concentration of labeled stearic and oleic acids could be determined in the liver. Comparisons could be made directly between NDFDA-treated rats, pair-fed control rats, and normally fed rats after correction for differences in fatty acid pool sizes. A considerable amount of information is already available concerning the fatty acid pool sizes in these three groups of rats (Olson, 1982). The low level of desaturase in the livers of NDFDA-treated rats suggests that the alterations in liver fatty acids are either due to changes in the turnover rate of cellular constituents containing fatty acids or in the rate of fatty acid transport to and from the liver. It is obviously not due to an increase in stearoyl-CoA desaturase, the amount of which is clearly decreased by NDFDA-treatment.

The decreased microsomal electron transport from NADH to the desaturase and other terminal oxidases in the NDFDA-treated rat liver is interesting. It does not appear to be due to either a reduction in the concentration of microsomal cytochrome b_5, or in its rate of reduction. It is possible that the rate of transport between cytochrome b_5 and the terminal oxidase (which is thought to be cytochrome P-450 in the absence of substrate for the desaturase) is markedly inhibited either due to a decrease in a reductase or to changes in the properties of the membrane. In regard to the latter possibility, perturbed lipid metabolism in the liver may lead to changes in the fluidity of the membrane as has been reported in different nutritional states (Kates and Pugh, 1980).

The apparent increase in cytochrome P-450 content of NDFDA-treated rats is unexpected since there was decreased inducibility of stearoyl-CoA desaturase by hyperalimentation, suggesting that the regulatory or synthetic mechanisms in the liver were damaged. Since these data were based on single animals, additional studies are warranted. However, the depressed cytochrome P-450 levels in pair-fed controls suggest that these data are valid. Unfortunately, the induction of cytochrome P-450 is not a reliable indication that the drug metabolizing pathway is more active. It would be desirable to obtain a measure of the overall activity of this pathway in NDFDA-treated and pair-fed rats by determining rates of metabolism of suitable substrates of the cytochrome P-450 oxidase system.

Because of the severity of NDFDA hepatotoxicity, liver regeneration (hypertrophy) may occur in response to cell destruction during initial exposure. Both the surgical removal of two-thirds of the liver and

treatment with CCl_4 are known to induce liver regeneration (Dorman and Webb, 1974). The relationship between liver regeneration and survivability at various dosages of NDFDA is of interest, since the LD_{50} may be related to the functional competence of reparative mechanisms of the liver.

In addition to overt hepatotoxicity, NDFDA may affect regulatory mechanisms in the surviving cells. Although nutritional induction of stearoyl-CoA desaturase is reduced, this could be the result of decreased absorption of the force-fed nutrients from the G.I. tract. The enzyme is also normally inducible by insulin (Prasad and Joshi, 1979) and assay for induction by this hormone would be a measure of both the intactness of the genetic regulation in the cell and the status of the insulin receptors in the membrane. Another hepatic enzyme which might be tested for inducibility is tyrosine transaminase, which is induced by insulin, glucagon, and glucocorticoids (McNamara and Webb, 1973). Additional studies of inducibility are necessary to establish the mechanism for the loss of inducibility and for the interference with regulatory control of vital metabolic processes.

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